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Characterization of a Bicistronic Retroviral Vector Composed of the Swine Vesicular Disease Virus Internal Ribosome Entry Site

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We cloned the 5′ nontranslated region (NTR) from the genome of swine vesicular disease virus (SVDV), a member of the family *Picornaviridae*, and used it to construct a bicistronic retroviral vector. The vector is characterized by coexpression of two genes from a single transcript. We found that inclusion of the 5′ NTR of SVDV did not negate the viral vector titer. Protein analysis indicated that the 5′ NTR could efficiently direct internal initiation, thus allowing the downstream gene to be translated. Translation of the internally initiated porcine growth hormone gene was about 30-fold less than that when the porcine growth hormone gene was at the upstream position in NIH 3T3 cells but was about equivalent to that in HeLa cells, implying that some cellular factors that stimulated internal initiation of the SVDV 5′ NTR are present in HeLa cells. However, in G418-selected clones, the Neo^r-encoding gene was expressed with equivalent efficiency either at a downstream position or at an upstream position in either NIH 3T3 or HeLa cells. Compared with the conventional double-gene vector or the U3-based vector, the bicistronic vector coexpressed two genes much more efficiently, owing to elimination of promoter interference. Furthermore, this type of vector infected and expressed the target genes efficiently in two primary cell lines, rat embryo and human skin fibroblast cells, which we tested. These experimental data suggest a better design for the retroviral vector and provide evidence that internal initiation of the SVDV 5′ NTR was stimulated cell specifically.

Retroviral vectors have been widely used in gene transfer, since they have broad host ranges (8, 32). In many situations it is essential to express two genes from a single proviral genome, one for the selectable marker to facilitate the isolation of a stable cell lineage and the other for the gene of interest. Conventional retroviral vectors include an exogenous promoter inserted at a site within the retroviral transcriptional unit. The upstream gene is expressed from the retroviral long terminal repeat (LTR), and the downstream gene is expressed from the internal promoter (9, 13). The problem with such a design is competitive interference between two promoters (9, 10, 24). As a result, one of these two genes may not be transcribed efficiently in individual isolates. Several approaches have been used to improve the vector design, such as the use of a self-inactivating vector (13) or a U3-based vector (4, 15). However, we have shown that the exogenous gene expressed from a double-gene vector, whichever is currently available, is never expressed as efficiently as from a single-gene vector (17).

Recently, the picornavirus 5′ nontranslated region (NTR) has been demonstrated to possess a specific secondary structure, thus allowing the ribosome to bind to it directly and initiate protein translation from a downstream AUG codon (see review in reference 19). This mechanism is different from the ribosomal scanning model that is considered valid for the translation of most eukaryotic cellular and viral mRNAs (20). By taking advantage of the property of the 5′ NTR of picornavirus, it is possible to construct a single-transcript type of retroviral vector which can simultaneously express two exogenous genes. In this way, only one transcript is expressed from the LTR promoter; thus, transcriptional interference of two active promoters can be avoided. Indeed, this has been documented in two reports demonstrating that insertion of the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) in a retroviral vector allows two cistrons to be expressed efficiently from a single transcript (1, 12). However, picornaviruses have been traditionally classified into several genera. The 5′ NTR sequences thought to be important for internal initiation of different genera are quite different and can be grouped together into three classes: (i) hepatitis A virus; (ii) cardioviruses and aphthoviruses, e.g., EMCV and foot-and-mouth disease virus; and (iii) enteroviruses and rhinoviruses, e.g., poliovirus and rhinovirus. As a result, the mechanism for initiation on cardiovirus and aphthovirus may be different from that for initiation on enterovirus and rhinovirus (19).

Swine vesicular disease virus (SVDV) is an enterovirus belonging to the family *Picornaviridae*. It causes an infectious disease in pigs that is characterized by the appearance of vesicles on the tongue, in the mouth, and on the feet and hocks (6, 25, 26). Like many picornaviruses, SVDV contains a very long 5′ NTR, 742 nucleotides in length, which contains nine potential translation start codons positioned before the codon that initiates the large open reading frame at nucleotide position 743 (18). Within the NTR, the predicted stem-loop structure, as described for the 5′ NTR of poliovirus, is conserved in SVDV (18, 19, 33). Therefore, it is very likely that the 5′ NTR of SVDV works as an IRES for protein translation.

In this study, we demonstrated the capability of internal initiation on the SVDV 5′ NTR through analysis of a bicistronic retroviral vector containing the sequence. In addition, we found that the internal initiation capability of the SVDV 5′ NTR was greatly enhanced in HeLa cells

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compared with that in NIH 3T3 cells. The expression efficiency of the IRES-based bicistronic retroviral vectors was also much higher than that of the conventional double-gene vector or the U3-based vector.

MATERIALS AND METHODS

Cell culture. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator. This experiment used the GP+E-86 ecotropic retrovirus packaging cell line (22) and the GP+*env*Am12 amphotropic packaging cell line (23) to produce recombinant retroviruses. NIH 3T3 cells were the major target cells used for analysis in this study. Primary human skin fibroblast cells were isolated from foreskin biopsies of a normal human. Rat embryo fibroblast cells (REF) were isolated from 17- to 18-day-old Norway brown rat embryos by the cold-trypsin method (11).

Retroviral vector construction. All vectors were derived from the N2 vector (2). The Tn*5* neomycin resistance (Neo^r) gene and a cDNA that codes for porcine growth hormone (pGH), a gift from W. C. Chang (National Taiwan University), were used as the tested genes in constructions. This pGH cDNA includes the 5′ nontranslated region, the signal peptide region plus the full coding region of pGH, and the 3′ nontranslated region but excludes the polyadenylation signal. Plasmid P (see Fig. 1) was constructed by replacing the Neo^r gene with the pGH cDNA fragment through blunt-end ligation to the N2 vector at the *Eco*RI site (2). The NP and PN plasmids were constructed by placing the pGH cDNA downstream or upstream of the Neo^r gene by blunt-end ligation to the N2 vector at the *Xho*I or *Eco*RI site, respectively. Such constructions thus put two genes within the same transcriptional unit. A 742-bp-long 5′ NTR cDNA fragment of SVDV was obtained by reverse transcription-polymerase chain reaction amplification of the viral RNA, which was prepared from the virus particles isolated from the stool of SVDV-infected pigs (30) and was kindly provided by H. S. Lai (National Taiwan University). The primer sequences used (5′-TTAAAACAGCCTGTGGGTTG-3′ and 5′-TTTAACGTATTGAGCGTTAT-3′) were adopted from the published data (18). This segment contains the putative IRES but excludes the authentic AUG initiation codon of SVDV. Plasmids NSP and PSN were constructed by insertion of the 5′ NTR cDNA fragment of SVDV between the Neo^r gene and the pGH cDNA on plasmids NP and PN, respectively. Plasmid PMX contained an internal metallothionein (MT) promoter cloned at the *Eco*RI site of the N2 vector. The pGH cDNA was placed under control of the LTR promoter, while the Neo^r gene was under control of the MT promoter. The last plasmid, CB, is a U3-based retroviral vector. The pGH cDNA was first cloned downstream of the immediate-early gene promoter of cytomegalovirus (CMV); the CMV-pGH cassette was subsequently inserted into the 3′ U3 region at the *Bam*HI site before the LTR promoter (4). A unique *Hin*dIII site was created at the 3′ end of CMV-pGH and could be used to check the duplication event of the 3′ U3 region after reverse transcription (see Fig. 2).

Virus production and infection. Virus was generated by transfecting 2×10^5 ecotropic GP+E-86 or amphotropic GP+*env*Am12 packaging cells with 20 μg of vector DNA by using the calcium phosphate precipitation procedure (14). For the P plasmid, which has no Neo^r gene, 18 μg of P plasmid DNA was cotransfected with 2 μg of pSV2neo DNA (used as a selectable marker) into the packaging cell lines. Transfected cells were selected with 0.8 mg of G418 per ml, and resistant colonies were pooled to form the virus-producing cells. Viruses were harvested 16 to 18 h after fresh medium was placed onto the nearly confluent virus-producing cells and were used to infect NIH 3T3, primary human fibroblast, and REF target cells. Infection was performed in the presence of 8 μg of Polybrene per ml. Since NIH 3T3 cells infected with P virus were not selectable, they were coinfected with 2 ml of pGH retrovirus and 2 μl of Neo^r virus (i.e., N2 virus) to obtain G418-resistant clones which were simultaneously infected with the pGH retrovirus (17). Selection was continued until colonies appeared. All of the G418-resistant clones (>1,000) were pooled for further analysis.

Southern and Northern (RNA) blot analyses. High-molecular-weight DNA was digested with restriction enzymes, electrophoresed on a 0.8% agarose gel, and electrotransferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, N.H.). The pGH-specific probe was used to hybridize the membrane. RNA was extracted from cultured cells by the acid guanidinium thiocyanate method (5). Ten micrograms of total RNA was subjected to glyoxal-dimethyl sulfoxide-agarose gel electrophoresis (31), transferred to the Nytran membrane, and hybridized with the pGH probe.

ELISA for pGH. Rabbit anti-pGH serum was obtained by immunizing rabbits intrasplenically (16) and was kindly provided by W. C. Chang. In the enzyme-linked immunosorbent assay (ELISA), standard pGH (a gift from International Mineral Corp.) at several known concentrations and culture medium samples were first coated onto 96-well plates. Rabbit antiserum (1:10,000 dilution) was added and allowed to react with pGH at 37°C for 1 h. After removal of the first antibody by four washes with phosphate-buffered saline (PBS), a second antibody, goat anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase, was added (1:3,000 dilution) for a further 1 h of incubation. The plates were washed again thoroughly with PBS, and 100 μl of substrate solution (2 mg of *o*-phenylenediamine dissolved in 1 ml of 1 M phosphate citrate [pH 5.0] plus 0.02% H_2O_2) was added for development. After 30 min, 100 μl of 1 M H_2SO_4 was added immediately to stop the reaction and the colors were measured by optical density at 492 nm. A standard pGH curve based on the optical density at 492 nm of a known pGH concentration was plotted, and the concentrations of pGH in our samples were obtained by interpolation from the standard curve.

Western blot (immunoblot) analysis. To analyze the pGH protein translated from the infected clones, cells were grown in a 10-cm dish until confluent and washed twice with PBS. A serum-free medium, Iscove's modified Dulbecco's medium, was used to refresh the cells overnight. The overnight-culture medium was collected, concentrated by Amicon Centriprep-10, and subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (21). Proteins were transferred from the gel to a nitrocellulose filter (3). The pGH protein was reacted with rabbit anti-pGH serum and subsequently with an alkaline phosphatase-conjugated second antibody, goat anti-rabbit immunoglobulin G. The blot was then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for a few minutes until color developed.

Quantitative assay of NPTII activity. Quantitative assay of neomycin phosphotransferase (NPTII) was done as described by Reiss et al. (29). Infected cells were harvested and adjusted to equal intensities (5×10^5 cells per 20 μl for NIH 3T3 cells and 7.5×10^5 cells per 20 μl for HeLa cells) with TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA). Crude cell

extract was obtained by sonication and separation from cellular debris by centrifugation. Twenty microliters of the extract was subjected to nondenaturing 10% polyacrylamide gel electrophoresis. The polyacrylamide gel was equilibrated in 100 ml of reaction buffer (67 mM Tris maleate [pH 7.1], 4.2 mM $MgCl_2$, 400 mM NH_4Cl) for 30 min, transferred onto a glass plate, and covered with a second gel made up of 1% agarose in the same reaction buffer containing the substrates (0.5 to 2 nM [γ-^{32}P]ATP and 20 μg of kanamycin sulfate per ml). After incubation for 30 min at room temperature, a sheet of Whatman P81 paper, two sheets of Whatman 3MM paper, a stack of blotting paper, and a 1-kg weight were put on top of the agarose gel. After 3 h, the P81 papers were removed, washed twice with 500 ml of hot water (80°C) and several times with cold water, dried, and exposed to X-ray film with an intensifying screen.

RESULTS

Efficient internal initiation and high viral titer from a 5′ NTR-containing bicistronic retroviral vector. The retroviral vectors used here were N2 vector derivatives (2). The LTR promoter was derived from the Moloney murine leukemia virus genome. The virus sequences retained in the vector consisted of the packaging signal and a pair of cryptic splicing signals. A full-length RNA that codes for an out-of-frame *gag* insert fusion protein, as well as a cryptically spliced mRNA that codes for the authentic protein of the inserted sequences, was detected (2). Four types of retroviral vectors were constructed (Fig. 1). The first type was a single-gene vector, P, which contained only the pGH cDNA in the retroviral vector and was not selectable. The second type was a bicistronic vector. NP and PN contained two exogenous genes, those for pGH and Neo^r, without the 5′ NTR segment of SVDV in the intercistronic region, whereas NSP and PSN contained a 742-bp fragment of the 5′ NTR of SVDV inserted between two cistrons. The third type was a conventional retroviral vector, PMX, which included a pGH cDNA driven by the LTR promoter and a Neo^r gene driven by an internal MT promoter within the LTR transcriptional unit. The last type was a U3-based retroviral vector, CB, in which the CMV-pGH expression cassette was inserted at the 3′ U3 region, thus allowing the cassette to be duplicated to the U3 region of the 5′ LTR after one cycle of reverse transcription (4, 15).

All of the plasmid DNAs except P were directly transfected into the GP+E-86 ecotropic packaging cell line, viruses collected from GP+E-86 cells were used to infect NIH 3T3 cells, and virus titers were determined. Since no selectable marker was included in vector P, the GP+E-86 cell line was cotransfected with 18 μg of vector DNA and 2 μg of pSV2neo DNA to generate a stable, virus-producing cell line. Similarly, NIH 3T3 cells were coinfected with P virus and Neo^r virus to produce a stable, G418-resistant cell line which simultaneously expressed pGH (17). Viruses with titers ranging around 10^5 CFU/ml, comparable to that of the virus from cells containing the N2 vector, were obtained from cells harboring vectors NP, NSP, and PSN, but only 10^3 CFU/ml was obtained from cells harboring vectors PMX and CB. No colonies of cells transfected or infected with the PN construct were observed, a result reproducibly obtained. The results therefore have two implications. (i) Expression of Neo^r gene from the downstream cistron of vector PN was not sufficient to confer G418 resistance on the GP+E-86 cell line. However, the 5′ NTR of SVDV contained in the intercistronic region of vector PSN immediately conferred sufficient G418 resistance to GP+E-86 and NIH 3T3 cells, suggesting that the 5′ NTR of SVDV could function as an IRES. (ii) Inclusion of the 742-bp SVDV 5′ NTR fragment within the retroviral vector did not adversely affect the virus titer, since the bicistronic vectors had titers similar to that of the single-gene vector. On the contrary, both the conventional retroviral vector and the U3-based vector had much lower titers, apparently owing to interference between two promoters (4, 10).

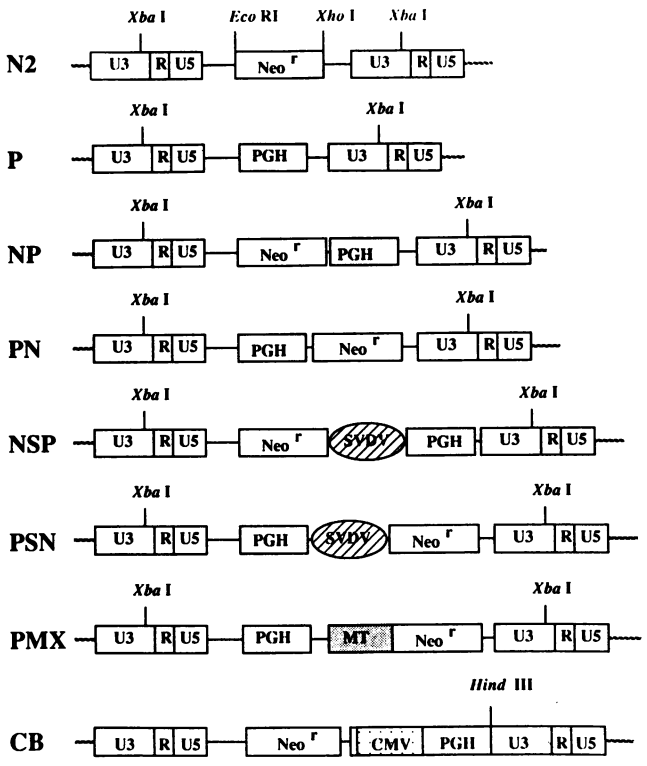

FIG. 1. Retroviral vectors. The N2 vector was described previously (2). All of the vectors shown were constructed on the basis of the backbone of N2. Abbreviations: PGH, the cDNA that codes for pGH; Neo^r, DNA for neomycin phosphotransferase; SVDV, the cDNA fragment of the 5′ NTR of SVDV; MT, the MT promoter; CMV, the immediate-early gene promoter of CMV. Names for the bicistronic vectors are based on the orders of DNA fragments. P stands for pGH, N stands for Neo^r, and S stands for the 5′ NTR of SVDV. PMX is a conventional retroviral vector with an MT-Neo^r cassette inserted at the *Xho*I site of N2; CB is a U3-based vector with a CMV-pGH cassette inserted at the *Bam*HI site of the 3′ U3 region of the N3 vector (4).

Expression of exogenous genes from the IRES-based bicistronic retroviral vector. The internal initiation capability of the 5′ NTR of SVDV was primarily demonstrated by the above-described experiment; we further performed detailed analysis to understand how it worked and whether it would affect the translation of the upstream cap-dependent cistron within the retroviral vector. To avoid bias due to the different expression efficiencies that resulted from different integration sites in individual clones, we pooled all of the G418-resistant colonies (>1,000) from each infection experiment for analysis. Figure 2A shows the results of Southern blot analysis of the proviral DNA present in the construct-infected clones. Restriction enzyme *Xba*I or *Hin*dIII was

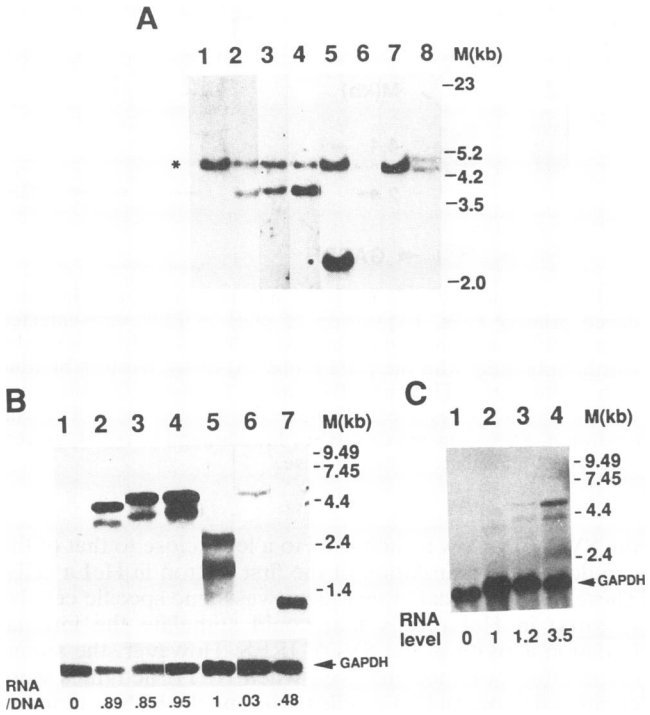

FIG. 2. Transcriptional rates of various retroviral vectors in infected cells. (A) Southern blot analysis of the proviral DNA in infected NIH 3T3 cells. Genomic DNAs extracted from infected cells were digested with either *Xba*I (lanes 1 to 6) or *Hin*dIII (lanes 7 and 8). Lanes 1 and 7 contained the NIH 3T3 cell control; the others contained cells infected with NP (lane 2), NSP (lane 3), PSN (lane 4), P (lane 5), PMX (lane 6), or CB (lane 8) virus, respectively. The asterisk indicates the position of the endogenous gene for mouse growth hormone, which was cross-hybridized with the pGH probe. M, markers. (B) Northern blot analysis of total RNA from virus-infected NIH 3T3 cells. RNA extracted from NIH 3T3 control cells (lane 1) or cells infected with NP (lane 2), NSP (lane 3), PSN (lane 4), P (lane 5), PMX (lane 6), or CB (lane 7) virus were analyzed. The filter was hybridized with a pGH probe and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, which was used as an internal control and whose position is indicated. Transcriptional rates were estimated by first adjusting the RNA level with the internal GAPDH control and then dividing the resulting RNA amount with the average DNA copy number shown in panel A, based on densitometric quantitation, and is expressed as the RNA/DNA ratio shown at the bottom of each lane. (C) Northern blot analysis of total RNA from infected HeLa cells. Lane 1 contained the HeLa cell control; the others contained cells infected with NP (lane 2), NSP (lane 3), or PSN (lane 4) virus. The relative RNA levels normalized to the GAPDH level are shown at the bottom.

used to detect the integrity of the proviral DNA. The results showed that there was no gross rearrangement of the proviral genome in any of the infected clones. Duplication of the chimeric U3 region was observed (Fig. 2A, lane 8) in CB virus-infected NIH 3T3 cells, as described previously (4). When normalized to the endogenous gene of the mouse growth hormone (indicated by an asterisk) which was cross-hybridized with the pGH probe, most of the proviral DNAs were present as one copy, on average, in the genome of the infected cells, except for PSN and P, which were present as two copies (lanes 4 and 5). Northern blot analysis of the RNA expressed from each vector is shown in Fig. 2B. All of the clones harboring constructs NP (lane 2), NSP (lane 3), PSN (lane 4), and P (lane 5) contained the full-length RNA,

TABLE 1. pGH production in infected cells

Vector	pGH production (ng/10^6 cells/day)[a]			
	NIH 3T3	HeLa	REF	Human skin fibroblast
NP	—[b]	—	ND[c]	ND
NSP	24	40	ND	ND
PSN	744[d]	40[d]	1,180	760
P	680[d]	ND	ND	ND
PMX	13	ND	ND	ND
CB	362	ND	ND	ND

[a] Overnight-culture medium was collected, and pGH protein was quantitated by ELISA (see Materials and Methods).
[b] —, the level of pGH protein was too low to be detected by ELISA.
[c] ND, not done.
[d] The level of pGH protein was divided by two since there are two copies of proviral DNA in the genome of this infected clone.

as well as the cryptically spliced RNA, characteristic of the N2 vector (2). The expression efficiency of vector NP, NSP, or PSN, after being adjusted with respect to the proviral DNA copy number, was equivalent to that of single-gene vector P, a result consistent with the viral titers obtained. However, the pGH RNA expressed from the PMX vector was dramatically reduced (lane 6), to only about 3% of that of the P vector. The pGH RNA expressed from the CMV promoter of the CB vector was about half of that of the P vector (lane 7). In cells containing the CB vector, the full-length RNA derived from the LTR promoter was strongly suppressed by the CMV promoter; thus, it was detectable only in a longer exposure of the Northern blot (data not shown). These data confirmed the problem of promoter interference inherent in the double-gene vectors, e.g., PMX and CB, and demonstrated the advantage of the IRES-based bicistronic vector.

The pGH protein translated from the mRNA derived from each vector was analyzed by ELISA and Western blotting, and the results are shown in Table 1 and Fig. 3A, respectively. As expected, the amount of pGH protein produced from the NP vector was too small to be detected (lane 2). On the contrary, pGH protein was produced from both NSP and PSN vectors but was recovered in 60-fold excess from the latter (lanes 3 and 4, respectively). If the RNA amount of individual clones was taken into consideration, the translational efficiency of the unselectable pGH gene from the upstream cistron was 30-fold higher than that from the downstream cistron (Fig. 3A and Table 1). Nevertheless, when the enzymatic activity of NPTII was measured, protein translation from the downstream cistron was almost as efficient as that from the upstream cistron after adjustment of the protein activity for the RNA amount (Fig. 3B, lanes 3 and 4). On the other hand, the translational efficiency of pGH protein from double-gene vector PMX or CB was more or less comparable to that from vector P after adjustment for the RNA level of each clone (Fig. 3A and Table 1).

These data therefore suggested that insertion of the SVDV IRES in the bicistronic retroviral vector did not affect the cap-dependent translation of the first cistron; the internal initiation efficiency from the SVDV IRES was only about 1/30 of that of the cap-dependent translation when the gene was not selectable but was increased to an equivalent level after the gene was selected.

Stimulation of the internal initiation activity of the SVDV IRES in HeLa cells. By using the IRES derived from EMCV in a bicistronic retroviral vector, Adam et al. (1) have

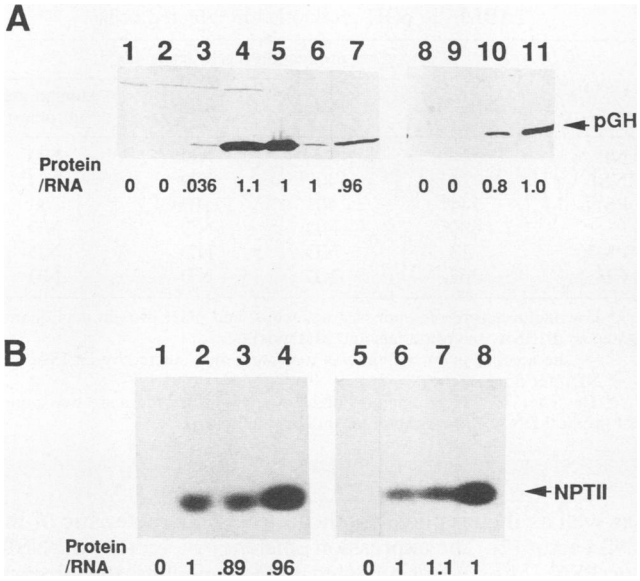

FIG. 3. Quantitation of protein expression from the retroviral vectors. (A) Western blot analysis of the pGH protein expressed from each vector. The pGH protein secreted into the supernatant of cultured cells was collected and analyzed by Western blotting (see Materials and Methods). The orders for lanes 1 to 7 and 8 to 11 are the same as those described in the legend to Fig. 2B and C, respectively. The position of the 22-kDa pGH protein is indicated. The translational efficiency of pGH from each vector (or cistron) is represented by the protein/RNA ratio shown at the bottom. (B) Assay of NPTII activity. The crude extracts of equal cell numbers (5×10^5 for NIH 3T3 cells and 7.5×10^5 for HeLa cells) were fractionated on a nondenaturing polyacrylamide gel. NPTII activity was assayed by in situ reaction of the polyacrylamide gel with an agarose gel containing the substrates kanamycin and [γ-^{32}P]ATP (see Materials and Methods). The position of the NPTII enzyme is indicated. Quantitation of enzyme activity was done by cutting out the spot from P81 paper and counting the radioactivity with a scintillation counter. The translational efficiency of NPTII is represented by the protein/RNA ratio shown at the bottom. Lanes 1 to 8 depict control cells (lanes 1 and 5) and NP (lanes 2 and 6), NSP (lanes 3 and 7), and PSN (lanes 4 and 8) virus-infected NIH 3T3 and HeLa cells, respectively.

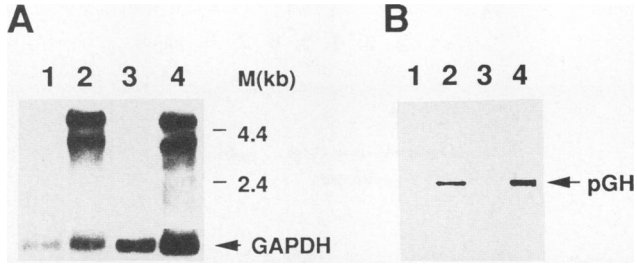

FIG. 4. Expression of the bicistronic retroviral vector in transduced primary cells. Expression of pGH in PSN virus-infected primary REF and human skin fibroblast cells was analyzed either by Northern blotting with the pGH probe (A) or by Western blotting with rabbit anti-pGH serum (B). Lanes: 1, human fibroblast cell control; 2, PSN virus-infected human fibroblast cells; 3, REF cell control; 4, PSN virus-infected REF. M, markers; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

previously shown that the downstream coding region was expressed at a fourfold lower level than that directed by the first cistron. This is in contrast to our finding of a 30-fold difference (Fig. 3A). Because the IRES sequence of EMCV is quite different from that of SVDV, the mechanisms of internal initiation of the two viruses could be quite different (19). It has been demonstrated that some specific cellular factors are present in HeLa cell extract that can stimulate the IRES function of poliovirus (7). It was therefore interesting to determine whether this would apply to the SVDV IRES and whether this may account for the dramatic reduction of translational efficiency of the internal initiation in our bicistronic vectors. NP, NSP, and PSN viruses were therefore used to infect HeLa cells. The RNA and protein were analyzed as described above, and the results are shown in Fig. 2C and 3, respectively. Interestingly, the pGH protein initiated from the IRES in the NSP vector was greatly increased relative to that from the first cistron of the PSN vector (Fig. 3A, lanes 10 and 11 versus lanes 3 and 4). Considering the different RNA amounts in the clones (Fig. 2C, lanes 3 and 4), the translational efficiency derived from the SVDV IRES was increased to a level close to that of the cap-dependent translation of the first cistron in HeLa cells. These results indicated that there was some specific cellular factor(s) in HeLa cells that could stimulate the internal initiation activity of the SVDV IRES. However, the stimulation effect was not observed when NPTII activities were compared between HeLa cells harboring the PSN vector and cells harboring the NSP vector (Fig. 3B, lanes 7 and 8 versus lanes 3 and 4).

Analysis of expression of the bicistronic vector in primary cells. To determine how the bicistronic vector is expressed in primary cells, REF and human skin fibroblast cells were infected with PSN virus. In both cases, the recombinant retrovirus infected the primary cells efficiently. Expression of pGH was analyzed at RNA and protein levels. Results showed that RNA was highly transcribed from the retroviral LTR promoter with the expected pattern in both fibroblast cells (Fig. 4A). The cap-dependent translation of pGH was also not affected by the presence of the SVDV IRES (Fig. 4B and Table 1). The secretion rates of pGH from REF and human skin fibroblast cells were estimated to be 1.18 and 0.76 μg/10^6 cells per day, respectively (Table 1).

DISCUSSION

The major concern of coexpressing two genes in a retroviral vector is often poor expression of one of them. This is due mainly to promoter interference (10), since most retroviral vectors contain two promoters for this purpose. Although the U3-based vector has been designed to overcome this problem (4, 15), promoter interference on the vector DNA still remains in the DNA-transfected packaging cells, resulting in lower titers of the recombinant viruses. The results we obtained strongly confirmed this notion. The discovery of the internal-initiation property of the 5′ NTR of picornavirus has allowed the translation of two genes from a single transcript (1, 12). Such a construct, as only one promoter is included, is devoid of promoter interference. In this study, we adopted the IRES derived from the SVDV genome to construct a bicistronic retroviral vector and successfully demonstrated its internal initiation capability. These results corroborated those of previous studies, in which an EMCV IRES was used (1, 12). Most importantly, the overall coexpression efficiency from the IRES-based vector was much higher than that from the conventional retroviral vector or from the U3-based vector (Fig. 3A). As

shown in Fig. 2B, the IRES-based vector was much more advantageous than the other two types of vectors, basically at the transcriptional level. Otherwise, the translational efficiency of pGH from the upstream position of the IRES-based vector was comparable to that from the LTR-driven cistron (vector PMX) or from the CMV-driven cistron (vector CB), representative of conventional vectors or U3-based vectors, respectively (4).

That our bicistronic vector differed from previous ones (1) in the difference of translational efficiency between two cistrons was probably a result of the different IRES sources used. Although both EMCV and SVDV are picornaviruses, they belong to different subgroups. It has been reported that cardiovirus-aphthovirus and enterovirus-rhinovirus are distinctly different not only in IRES sequences but also in translation characteristics (19). For example, foot-and-mouth disease virus and EMCV RNAs are both translated with very high efficiency and high accuracy in the rabbit reticulocyte lysate. On the other hand, poliovirus is translated very inefficiently and inaccurately in the rabbit reticulocyte lysate but efficiently and accurately in HeLa or L-cell extracts (27). Addition of HeLa extracts to reticulocyte lysate increases the fidelity and efficiency of poliovirus RNA translation (7), implying that specific cellular factors present in HeLa cells are required for efficient initiation on poliovirus RNA. Such a cellular factor, a 57-kDa protein, was recently identified from HeLa cell extracts (28). Because SVDV is an enterovirus, translation initiation is more similar to that of poliovirus. Indeed, we found that the translational efficiency of pGH from the IRES-initiated cistron was greatly increased when vectors were transduced into HeLa cells (Fig. 3A), implying that the cellular factors were cell type specific. However, when a Neo^r gene was assayed, it was found to be translated with equivalent efficiency from either the LTR-directed cistron or the IRES-directed cistron in both NIH 3T3 and HeLa cells. No significant stimulation of internally initiated NPTII activity was observed in HeLa cells either. The different results obtained with regard to pGH protein and NPTII enzymatic activity may be due to the selection pressure exerted on the infected clones. As a result, NPTII activity was forced to increase to a level sufficient to render cells viable; the activity measured may not reflect a real translational effect of different cistrons. Alternatively, the translational effect may vary from target gene to target gene, although we think this is less likely. To exclude the latter possibility, more nonselectable genes could be tested with this bicistronic vector in both NIH 3T3 and HeLa cells. We are currently performing this experiment.

In conclusion, our study suggests that the IRES-based vector is a better retroviral vector for coexpression of two genes with higher efficiency. Because the retroviral vector transduces genes into the chromosome through its unique integration mechanism, the RNA transcribed from an IRES-based vector is derived solely from the LTR promoter and no internally initiated mRNA could account for translation of the second cistron (1, 12; Fig. 2B). This system is therefore a powerful tool with which to study the internal initiation mechanism in a stable cell line and to identify any other potential IRES. It is also useful for studying the different mechanisms of internal initiation employed by different picornaviruses.

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